Clontech Laboratories, Inc.

Guide-it™ sgRNA *In Vitro* Transcription and Screening Systems User Manual

Cat. Nos. 631438, 631439 & 631440
(051415)

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I. Introduction

A. Summary

Cas9/CRISPR is a breakthrough genome editing technology that permits site-specific cleavage of DNA targets in the genomes of various organisms and mammalian cells. Disruptive mutations are created at those cleavage sites by error prone repair or by using homologous recombination to change or insert sequences. The power of this technology derives from its simplicity, since all it requires is a Cas9 nuclease enzyme combined with a single guide RNA (sgRNA) that determines its target specificity. Each sgRNA is designed by the user to contain a short sequence homologous to a target sequence of choice, with which it directs the Cas9 nuclease to introduce a double-stranded break at that sequence (Figure 1).

This user manual describes how to use the following kits:

- The **Guide-it sgRNA *In Vitro* Transcription Kit** (Cat. No. 631438) is used to produce high yields of single guide RNAs (sgRNAs) via *in vitro* transcription.
- The **Guide-it sgRNA Screening Kit** (Cat. No. 631440) enables the user to test the efficacy of different single guide RNAs (sgRNAs) *in vitro* prior to using them in studies involving Cas9-mediated gene editing. With this kit, a template containing a sgRNA target site is created by PCR, then combined with an sgRNA that you wish to test and Cas9 nuclease. The efficiency with which Cas9 nuclease cleaves the template can be measured using agarose gel electrophoresis.
- The **Guide-it Complete sgRNA Screening System** (Cat. No. 631439) is a combination of both kits described above and is used to synthesize and test the efficacy of single guide RNAs (sgRNAs). The kit contains all the components needed to *in vitro* transcribe sgRNAs and test their efficacy against a PCR target fragment using recombinant Cas9 nuclease.

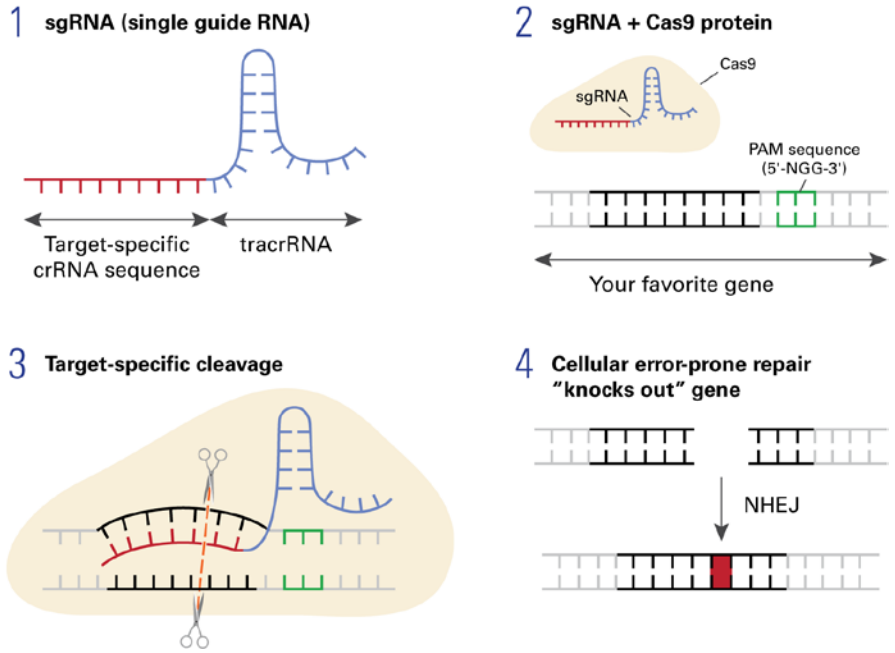

Figure 1. Using Guide-it technology to perform genome editing. A single guide RNA (sgRNA) consists of a crRNA sequence that binds to a specific DNA target, and a tracrRNA sequence that binds to Cas9 protein. When an sgRNA engineered to contain a 20 nucleotide (nt) target sequence of interest binds to a recombinant form of Cas9 protein that has double-stranded DNA endonuclease activity, the resulting complex will produce target-specific double-stranded cleavage. Cellular repair, which is error-prone, will take place at the cleavage site, and may result in a mutation that can "knock out" a gene.

B. Protocol Overview

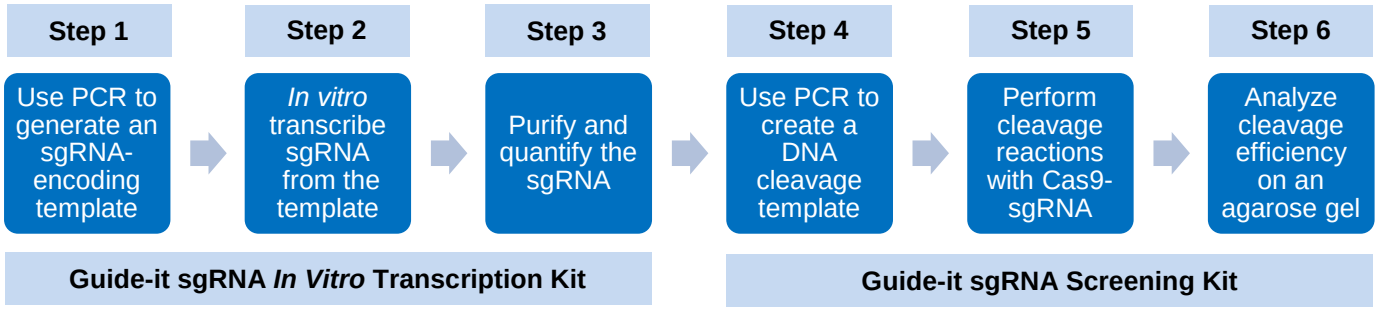

Figure 2. The Guide-it Complete sgRNA Screening System protocol.

The Guide-it Complete sgRNA Screening System can be used to synthesize and test the efficacy of single guide RNAs (sgRNAs) as follows (Figure 2):

1. Generate a DNA template that contains your sgRNA encoding sequence under the control of a T7 promoter by performing a PCR reaction with the included Guide-it Scaffold Template and a primer you design (Sections IV.A–B).
2. *In vitro* transcribe this template with the included Guide-it T7 Polymerase Mix to create an sgRNA containing your target sequence (Section IV.C).
3. Purify your sgRNA using digestion with DNAse I (supplied by the user), phenol:chloroform extraction, and ethanol precipitation; and measure its concentration using a NanoDrop 2000 spectrophotometer (Section IV.D).
4. Create a cleavage template for screening your purified sgRNA by amplifying a 2 kb fragment of genomic DNA that contains your target sequence in an asymmetric position that will produce two cleavage fragments of unequal size (Section V.A).
5. Perform a cleavage reaction on this template using your purified sgRNA in combination with the included Guide-it Recombinant Cas9 Nuclease (Section V.B).
6. Analyze the efficiency of your cleavage reactions on an agarose gel to determine if your target sequence was successfully incorporated (Section V.B).

II. List of Components

- Store Control sgRNA and Guide-it Recombinant Cas9 Nuclease at –70°C.
- Store all other components at –20°C.
- Avoid repeated freeze/thaw cycles.

Guide-it sgRNA *In Vitro* Transcription Kit (Cat. No. 631438)

- 1 each Guide-it sgRNA *In Vitro* Transcription Components
  (Cat. No. 631441; 10 rxns; Not sold separately)
  - 50 µl Guide-it Scaffold Template (20 ng/µl)
  - 70 µl Guide-it *In Vitro* Transcription Buffer
  - 30 µl Guide-it T7 Polymerase Mix (33 U/µl)
  - 500 µl RNase Free Water
- 1 each NucleoSpin Gel and PCR Clean-Up (Sample)
  (Cat. No. 740609.10; 10 preps; Not sold separately)
- 1 each High Yield PCR EcoDry™ Premix (Cat. No. 639278; 24 rxns)

Guide-it sgRNA Screening Kit (Cat. No. 631440)

- 1 each Guide-it Recombinant Cas9 Nuclease
  (Cat. No. 631442; 30 rxns; Not sold separately)
  - 30 µl Guide-it Recombinant Cas9 Nuclease (500 ng/µl)

    NOTE: Do not freeze/thaw Guide-it Recombinant Cas9 Nuclease more than 3 times. We recommend aliquoting it into multiple vials to avoid repeated freeze/thaw cycles.
  - 30 µl 10X Cas9 Reaction Buffer
  - 30 µl 10X BSA
  - 500 µl RNase Free Water
  - 10 µl 2 kb Control Fragment (40 ng/µl)
  - 6 µl Control sgRNA (20 ng/µl)
- 1 each NucleoSpin Gel and PCR Clean-Up (Sample)
  (Cat. No. 740609.10; 10 preps; Not sold separately)
- 1 each High Yield PCR EcoDry Premix (Sample) (Cat. No. 639279; 8 rxns)

Guide-it Complete sgRNA Screening System (Cat. No. 631439)

- 1 each Guide-it sgRNA *In Vitro* Transcription Components
  (Cat. No. 631441; 10 rxns; Not sold separately)
  - 50 µl Guide-it Scaffold Template (20 ng/µl)
  - 70 µl Guide-it *In Vitro* Transcription Buffer
  - 30 µl Guide-it T7 Polymerase Mix (33 U/µl)
  - 500 µl RNase Free Water
- 1 each Guide-it Recombinant Cas9 Nuclease (Cat. No. 631442; 30 rxns; Not sold separately)
  - 30 µl Guide-it Recombinant Cas9 Nuclease (500 ng/µl)
  - 30 µl 10X Cas9 Reaction Buffer
  - 30 µl 10X BSA
  - 500 µl RNase Free Water
  - 10 µl 2 kb Control Fragment (40 ng/µl)
  - 6 µl Control sgRNA (20 ng/µl)
- 1 each NucleoSpin Gel and PCR Clean-Up (Sample)
  (Cat. No. 740609.10; 10 preps; Not sold separately)
- 1 each High Yield PCR EcoDry Premix (Cat. No. 639278; 24 rxns)

III. Additional Materials Required

The following materials are required but not supplied:

- RNase free DNase I (we recommend Takara® Recombinant DNase I (RNase-free), Cat. No. 2270A)
- Phenol:chloroform: isoamyl alcohol (25:24:1), saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma, Cat. No. P2069-100ML)
- 3M sodium acetate, pH 5.0
- Isopropanol
- NucleoSpin Tissue kit (Cat. No. 740952.50)
- NanoDrop 2000 spectrophotometer (Thermo Scientific, Cat. No. ND-2000)

IV. *In Vitro* Transcription of an sgRNA Containing the Desired Target Sequence

Create an sgRNA containing your target sequence as follows:

1. Using the included Guide-it Scaffold Template and a primer you design (Section IV.A), PCR-amplify a DNA template that contains your sgRNA encoding sequence under the control of a T7 promoter (Section IV.B).
2. Perform an *in vitro* transcription reaction with the PCR product from Step 1 to generate your sgRNA (Section IV.C), then purify and quantify the newly synthesized sgRNA (Section IV.D).

A. PCR Primer Design Guidelines

Use the following guidelines to design a forward primer to be used in a PCR reaction with the included Guide-it Scaffold Template to create a DNA template for *in vitro* transcription of your sgRNA. This primer should contain the T7 promoter sequence, followed by your sgRNA targeting sequence of interest, and the Guide-it Scaffold Template-specific sequence (Figure 3).

1. Choosing the correct DNA target sequence

Choose the DNA target sequence that will correspond to your actual sgRNA targeting sequence as shown in Figure 3, Panel A, according to the following guidelines:

a. The DNA target sequence you choose must end with the proto-spacer adjacent motifs (PAM) sequence, NGG, on its 3' end. Only DNA sequences that are 20 nucleotides upstream of the PAM sequence can be targeted using Cas9/CRISPR.

b. The PAM sequence itself is NOT part of the sgRNA sequence.

2. Designing a 69 nt forward PCR primer

The forward (sense) primer must contain the following 3 sequence elements, as shown in Figure 3, Panel B.

a. T7 promoter sequence (28 nt at the 5' end of the primer): This inserts the sequence GGG at the 5' end of the sgRNA.

b. Actual sgRNA targeting sequence (20 nt in the middle section of primer): Any sgRNA sequence can be used; the 5' G that is required for expression of sgRNA from the U6 promoter is not required for this *in vitro* transcription template.

c. Scaffold Template-specific sequence (21 nt at the 3'end of the primer)

NOTES:

- A reverse (antisense) primer is already premixed with the Guide-it Scaffold Template.
- The forward primer should be subjected to salt-free purification following synthesis and diluted to a concentration of 10 µM in PCR-grade water.

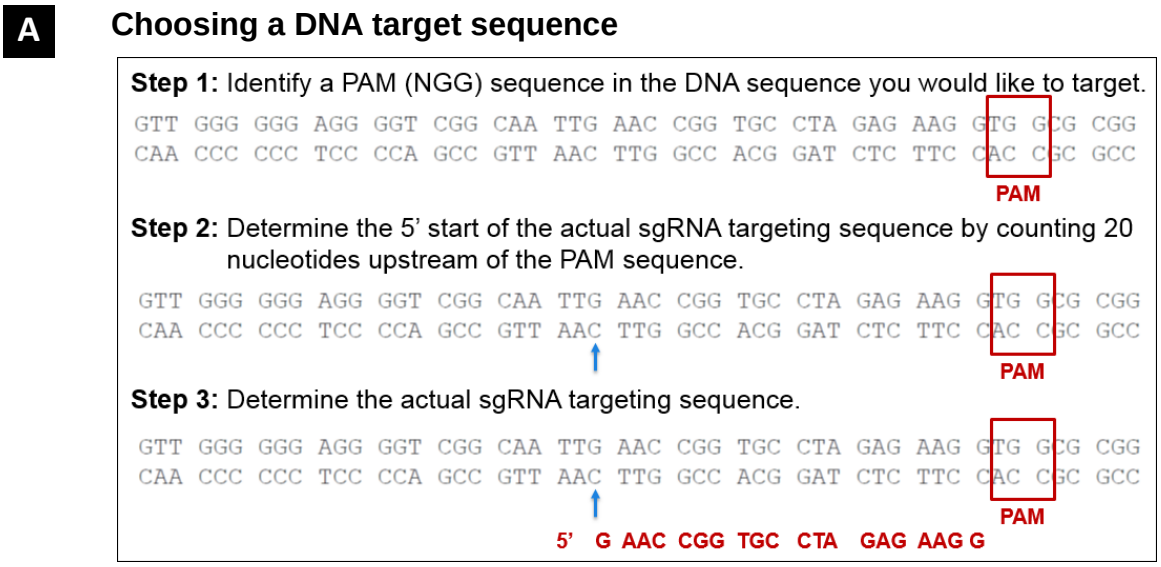

Figure 3. Designing a forward PCR primer to generate a DNA template for an sgRNA containing your target sequence. Panel A. Choose the DNA target sequence that will correspond to your actual sgRNA targeting sequence. **Panel B.** Design the forward primer to create the *in vitro* transcription template you will use to generate your sgRNA. (**NOTE:** The T7 promoter sequence and the Scaffold Template-specific sequence do **not** change.)

B. Protocol: Amplification of the sgRNA-Encoding Template

1. Combine 5 µl of Guide-it Scaffold Template with 1 µl of your forward primer designed according to the guidelines in Section IV.A (at a concentration of 10 µM), dilute with RNase-free water to a final volume of 25 µl, and vortex to mix.
2. Add the entire 25 µl from Step 1 to one tube of High Yield PCR EcoDry Premix, cap the tube, and perform thermal cycling using the following program:
   - 95°C 1 min
   - 33 cycles:
     95°C 30 sec
     68°C 1 min
   - 68°C 1 min
3. Analyze 5 µl of your resulting PCR fragment on a 1.8 % agarose gel. You should obtain a single ~140 bp band. Purify your fragment using the included NucleoSpin Gel and PCR Clean-Up kit and measure its concentration before proceeding to Section IV.C.

C. Protocol: *In Vitro* Transcription of the sgRNA-Encoding Template

1. Use your purified PCR fragment from Section IV.B to set up an *in vitro* transcription reaction:

Component	Volume
PCR Fragment (100 ng)	1–10 µl
Guide-it *In Vitro* Transcription Buffer	7 µl
Guide-it T7 Polymerase Mix	3 µl
RNase Free Water	0–9 µl*
Total volume per reaction	**20 µl**

*Depending on how much water is needed to bring the total volume to 20 µl.

2. Incubate at 42°C for 1 hr, then proceed to Section IV.D.

D. Protocol: Purification and Quantification of *In Vitro*-Transcribed sgRNA

This section describes how to purify your sgRNA using DNAse I digestion, phenol:chloroform extraction, and ethanol precipitation.

1. Add 2 µl of RNase free DNase I (Section III) to the entire 20 µl of the *in vitro* transcription reaction from Section IV.C and incubate at 37℃ for 0.5 hr.

   NOTE: The *in vitro* transcription reaction may be incubated for up to 1 hr.

2. Add RNase Free Water to the reaction mixture to a final volume of 100 µl.
3. Add 100 µl of phenol:chloroform: isoamyl alcohol (25:24:1), saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Section III) to the diluted reaction mixture from Step 2 and vortex well. Centrifuge at 12,000 rpm for 2 min at room temperature.
4. Transfer the supernatant to a new tube, add an equal volume of chloroform, and vortex well. Centrifuge at 12,000 rpm for 2 min at RT.
5. Transfer the supernatant to new tube, add 1/10 volume of 3M sodium acetate (Section III) and an equal volume of isopropanol (Section III), and vortex well. Incubate for 5 min at room temperature, then centrifuge at 15,000 rpm for 5 min at room temperature.
6. Remove the supernatant carefully. Rinse the pellet with 80% ethanol and centrifuge at 15,000 rpm for 5 min at room temperature.
7. Air dry the pellet for about 15 min and resuspend in 20 µl of RNase Free Water.
8. Use 1 µl of the resuspended sgRNA pellet from Step 7 to measure the yield of sgRNA using a NanoDrop 2000 spectrophotometer as described in the NanoDrop 2000 user manual. The expected yield of sgRNA is approximately 2–6 µg.

   IMPORTANT: Store the resuspended sgRNA pellet at –80℃ if it is not used immediately. It can be stored at –80°C for up to 6 months.

V. Screening sgRNAs for Effective Cleavage of their Targets

This section describes how to create a cleavage template (Section V.A) from your DNA of interest that contains your target sequence and how to use it to screen your sgRNA for effective target cleavage (Section V.B).

A. Protocol: Creating a Cleavage Template

1. Design the Primers

Design primers to amplify your target sequence within a ~2 kb amplicon, with the sgRNA target sequence located asymmetrically within the amplicon (Figure 4). This will enable the cleavage reaction with the Cas9-sgRNA complex (Section V.B) to generate two fragments of unequal size (differing by more than 500 bp) that are clearly separated on an agarose gel.

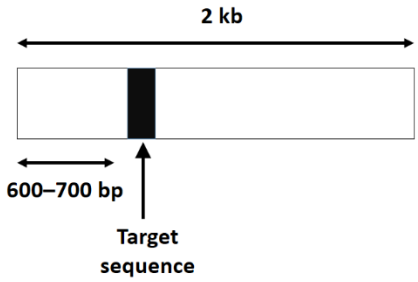

Figure 4. Designing a target DNA cleavage template.

2. Prepare the Template

The amplification reaction requires a DNA template containing your target sequence, which typically consists of purified genomic DNA. We recommend using the NucleoSpin Tissue kit (Section III) to purify your genomic DNA.

3. Assemble a Primer/Template Mix

Each tube of High Yield PCR EcoDry Premix requires the addition of PCR-grade water, primers, and template in a total volume of 25 µl. Assemble a mix containing these components in a volume sufficient for the number of PCR reactions you plan to perform, plus an additional reaction to compensate for pipetting errors.

Component	Volume
Genomic DNA (50 ng)	1–23 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
RNase Free Water	0–22 µl*
Total volume per reaction	**25 µl**

*Depending on how much water is needed to bring the total volume to 25 µl.

4. Reconstitute the Premix

a. Pipette 25 µl of the primer/template mix into each High Yield PCR EcoDry Premix tube.

b. Pipette up and down several times to dissolve each pellet, then cap the tubes.

c. Briefly spin the tubes in a microcentrifuge, and begin thermal cycling using the guidelines provided in Step 5.

5. **Recommended Cycling Parameters**

   Use the following guidelines when setting up your initial experiments. These are general guidelines—the optimal parameters may vary.

   - 95°C 1 min
   - 30 cycles:
     95°C 30 sec
     68°C 3 min
   - 68°C 3 min

6. Analyze your PCR product on an agarose gel, purify it using the included NucleoSpin Gel and PCR Clean-Up kit and measure its concentration before proceeding to the cleavage reaction in Section V.B.

B. Protocol: Using a Cleavage Reaction to Screen your sgRNA

Use this protocol to screen your cleavage template from Section V.A for your target sequence.

1. Set up a cleavage reaction containing your experimental sgRNA sample (specific for your target; from Section IV.D, Step 8) and your experimental cleavage template (~2 kb long, containing your target sequence; from Section V.A, Step 6), in parallel with a positive control reaction containing the included 2 kb Control Fragment and Control sgRNA (Section II):

Component	Reagent Volume (µl per sample)	
	Experimental Cleavage Reaction	Positive Control Cleavage Reaction
Experimental Cleavage Template (100 ng total)	1–6 µl	—
Experimental sgRNA Sample (20 ng total)	1–6 µl	—
2 kb Control Fragment (40 ng/µl)	—	2.5 µl
Control sgRNA (20 ng/µl)	—	1 µl
Guide-it Recombinant Cas9 Nuclease	1 µl	1 µl
10X Cas9 Reaction Buffer	1 µl	1 µl
10X BSA	1 µl	1 µl
RNase-free PCR-grade water	0–5 µl*	3.5 µl*
Total Volume	10 µl	10 µl

*Depending on how much water is needed to bring the total volume to 10 µl.

2. Incubate at 37°C for 1 hr.
3. Stop the reaction by incubating at 70°C for 10 min.
4. Analyze the entire 10 µl reactions on a 1% TAE agarose gel alongside a negative control (100 ng of uncleaved 2 kb Control Fragment). See Figure 5 for typical results.
   - Fragment size will depend upon site of cleavage. We recommend target sequence placement such that cleavage will result in clear separation of the products (i.e., a size difference between the expected fragments of greater than 500 bp; see Section V.A, Step 1).
   - In most cases where the cleavage efficiency is <100%, three bands should be observed, including uncut species as well as two bands from the cleaved product. The negative control lane (containing the uncut 2 kb Control Fragment) should contain only a single band, at 2 kb.
   - When the cleavage efficiency is 100%, only two bands will be observed (since no uncut fragment will be present).

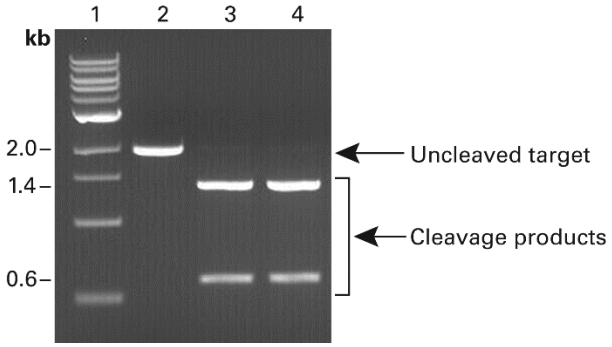

Figure 5. Analysis of cleavage products. An sgRNA sequence was synthesized and tested against its target in human chromosome 10. A 2 kb fragment of chromosome 10 was PCR-amplified, and an sgRNA designed to cleave the amplified sequence was in vitro-transcribed—using the Guide-it Complete sgRNA Screening System (Cat. No. 631439). The chromosome 10 fragment, the sgRNA, and recombinant Cas9 enzyme (also included in the kit) were combined in an in vitro cleavage reaction according to the protocol. Agarose gel analysis indicated that 10 ng (Lane 3) and 20 ng (Lane 4) samples of sgRNA were each sufficient for 100% cleavage of the target fragment. Lane 2 shows the untreated/uncleaved control.

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